



(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:

10.07.2002 Bulletin 2002/28

(51) Int Cl.7: C12N 15/12, C07K 14/51,
A61K 38/18, A61L 27/22,
A61L 27/54

(21) Application number: 01201555.8

(22) Date of filing: 07.04.1989

(84) Designated Contracting States:

AT BE CH DE FR GB IT LI LU NL SE

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(30) Priority: 08.04.1988 US 179406

15.08.1988 US 232630

23.02.1989 US 315342

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(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:

96200044.4 / 0 723 013

89904959.7 / 0 372 031

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Remarks:

This application was filed on 27 - 04 - 2001 as a divisional application to the application mentioned under INID code 62.

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(54) Biosynthetic osteogenic proteins and osteogenic devices containing them

(57) Disclosed are 1) osteogenic devices comprising a matrix containing osteogenic protein and methods of inducing endochondral bone growth and cartilage in mammals using the devices; 2) amino acid sequence data, amino acid composition, solubility properties,

structural features, homologies and various other data characterizing osteogenic proteins, 3) methods of producing osteogenic proteins using recombinant DNA technology, and 4) osteogenically and chondrogenically active synthetic protein constructs.

FIG. 1-1

		VG1 DPP OPI CBMP2		CBMP3	beta-	TGF-	MIS	alpha::	consensus	choices
COP1	COP5	a)	b)	(bov)	Inhibin	beta	Inhib	beta		
COP3	COP7					1 2 3:				
COP4		C	C	C	C	C C C	C	C C	C	c
		K	R	K	R	A C C	C	A H	k, r	
<P P	P P P	K	R	K	R	R K R	V L V	L R	k, r	
<N N	N N N	R	H	H	H	R K Q	R	R V	h, k, r, q	
<G G	G G G	H	S	E	P	Y Q Q	Q P P	E A	p, s, e, q	
L L	L L L	L	L	L	L	L F F	L	L L	l	
Y Y	Y Y Y	Y	Y	Y	Y	K F F	Y	S N	y, f	
V V	V V V	V	V	V	V	V V I	I	V I	v, i	
D D	D D D	E	D	S	D	D S D	D	D S	d, e, s	
F F	F F F	F	F	F	F	F F F	F	L F	f	
Q Q	S S S	S	S	R	S	A K R	R K R	R Q	k, r, s	
R R							R Q	A		
D D	D D D	D	D	D	D	D D	D	E E	d	
V V	V V V	V	V	V	V	I I I	L	R L	v, l, i	
G G	G G G	G	G	G	G	G G G	G	S G	g	
W W	W W W	W	W	W	W	W W W	W	W	w	
D D	D D N	D	Q	N	N	S N N	K	E	q, n, d, e, s	
D D	D D D	N	D	D	D	E D D	-	R	d, e, n	
W W	W W W	W	W	W	W	W W W	W	W	w	
I I	I I I	V	I	I	I	I I I	I V	V I	i, v	
I V	V V V	I	V	I	V	I I I	H	L V	i, v	
A A	A A A	A	A	A	A	S A A	E	I Y	a, s	
P P	P P P	P	P	P	P	P P P	P	P P	p	
V P	P P P	Q	L	E	P	K S T	K	E P	e, q, p*	
D G	G G G	G	G	G	G	S G G	G	T S	g, s	
F Y	Y Y Y	Y	Y	Y	Y	F Y Y	Y	Y F	y, f	
D Q	Q H H	M	D	V	H	D H Y	H N Y	Q I	h, d, n, q, y*	
A A	A A A	A	A	A	A	A A G	A	A F	a	

FIG. 1-2

FIG. 1-3

N	N	N	S	S	:	S	N	F	S	:	A	R	R	Q	T	T	Q	S	:	n,s*	
M	M	M	V	V	:	I	N	N	I	N	V	G	G	H	I	H	A	R	L	:	i,v,l,
N	N	N	N	N	:	N	N	N	N	N	N	N	N	N	N	N	G	G	L	:	n
P	P	P	S	S	:	P	P	P	S	:	G	P	P	G	E	E	A	P	G	:	p,s,a
G	G	G	:	E	G	:	E	:	G	:	P	F	A	N	T	K	A	L	G	:	g,e
K	K	K	K	K	:	Q	K	T	K	S	:	I	P	S	A	S	A	R	:	k,q,t,s	
V	V	V	I	I	:	P	V	P	K	5	:	P	C	C	S	S	S	P	:	i,v,l,	
P	P	P	P	P	:	P	V	P	K	5	:	P	C	C	C	C	C	P	:	p,s,s	
P	P	P	A	A	:	P	C	K	A	5	:	P	C	C	C	C	C	P	:	k	
C	C	C	C	C	:	C	C	C	C	5	:	P	C	C	C	C	C	P	:	a,p,s	
C	C	C	C	C	:	C	C	C	C	5	:	P	C	C	C	C	C	P	:	c	
V	V	V	V	V	:	C	C	C	C	5	:	P	C	C	C	C	C	P	:	v,a,i	
P	P	P	P	P	:	P	P	P	P	5	:	P	C	C	C	C	C	P	:	p,s,a	
T	T	T	E	E	:	T	T	T	T	5	:	P	C	C	C	C	C	P	:	t,e,d	
E	E	E	E	E	:	T	T	T	T	5	:	P	C	C	C	C	C	P	:	q,e,d,k*	
I	I	I	L	L	:	L	L	L	L	5	:	P	C	C	C	C	C	P	:	l,m	
S	S	S	S	S	:	S	S	S	S	5	:	P	C	C	C	C	C	P	:	s,n,e,d	
A	A	A	A	A	:	P	S	A	A	5	:	P	C	C	C	C	C	P	:	a,s,p,t	
I	I	I	I	I	:	S	V	A	A	5	:	P	C	C	C	C	C	P	:	i,l,m,v	
S	S	S	S	S	:	S	I	S	I	5	:	P	C	C	C	C	C	P	:	s,a,p,t	
M	M	M	M	M	:	M	S	M	S	5	:	P	C	C	C	C	C	P	:	v,m,i,l	
L	L	L	L	L	:	L	M	A	M	5	:	P	C	C	C	C	C	P	:	l,v,i	
Y	Y	Y	Y	Y	:	F	Y	L	Y	5	:	P	C	C	C	C	C	P	:	y,f	
L	L	L	L	L	:	F	Y	L	Y	5	:	P	C	C	C	C	C	P	:	f,l,y	
D	D	D	D	D	:	D	N	D	D	5	:	P	C	C	C	C	C	P	:	d,n	
E	E	E	E	E	:	D	N	D	D	5	:	P	C	C	C	C	C	P	:	d,e,	
N	N	N	N	N	:	D	N	D	D	5	:	P	C	C	C	C	C	P	:	n,g,y*	
E	E	E	E	E	:	D	N	D	D	5	:	P	C	C	C	C	C	P	:	d,e,s,t	

FIG. 1-4

K	K	K	K	:	N	T	N	K	:	N	N	N	P	:	I	G	:	n,k	
V	V	V	V	:	V	V	V	V	:	V	I	V	K	V	S	G	:	v,i	
V	V	V	V	:	V	V	I	V	:	V	I	V	E	I	A	Y	:	v,i	
L	L	L	L	:	L	L	L	L	:	L	K	K	Q	L	H	S	F	:	l
K	K	K	K	:	R	K	K	K	:	K	K	R	L		H	K	Y	:	k,r
N	N	N	N	:	H	N	K	N	:	V	D	D	V		V	K	Y	:	n,d,k*
Y	Y	Y	Y	:	Y	Y	Y	Y	:	Y	I	V	P	S	P	E	Y	:	y
Q	Q	Q	Q	:	E	Q	R	Q	:	P	Q	P					TVP	:	q,e,p,r
E	E	E	E	:	N	E	N	D	E	N	N	N	N		N	N		:	n,d,e
M	M	M	M	:	M	M	M	M		M	M	M	M		M	L		:	m
V	V	V	V	:	A	T	M	V		T	I	I	I	I	V	L		:	v,i,t,a
V	V	V	V	:	V	V	V	V		V	V	V	V		V	A	T	:	v
E	E	E	E	:	D	V	R	E		E	E	E	R	K	K	Q	H	:	e,d,r,k,
G	G	G	G	:	E	G	A	G		S	E	E	S	C	C	A	C	:	g,a,s,e
C	C	C	C	:	C	C	C	C		C	C	C	C	K	C	A	C	:	c
G	G	G	G	:	G	G	G	G		H	G	G	C	C	C	G	C	:	g,h
C	C	C	C	:	C	C	C	C		R	C	S	A	S	R	I		:	c
R	R	R	R	:	R	R	C	H										:	r,b,s,a.

Description

[0001] This invention relates to osteogenic devices, to synthetic genes encoding proteins which can induce osteogenesis in mammals and methods for their production using recombinant DNA techniques, to synthetic forms of osteogenic protein, and to bone and cartilage repair procedures using osteogenic device comprising the synthetic proteins.

[0002] Mammalian bone tissue is known to contain one or more proteinaceous materials, presumably active during growth and natural bone healing, which can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

[0003] The developmental cascade of bone differentiation consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) *Collagen Rel. Res.* 1:209-226).

[0004] Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) *Proc. Natl. Acad. Sci. USA* 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone *in vivo*.

[0005] This putative bone inductive protein has been shown to have a molecular mass of less than 50 kilodaltons (kD). Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) *Proc. Natl. Acad. Sci. USA* 80:6591-6595).

[0006] The potential utility of these proteins has been widely recognized. It is contemplated that the availability of the pure protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive procedures.

[0007] The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. (*Proc. Natl. Acad. Sci. USA* (1987) 80). Urist et al. (*Proc. Soc. Exp. Biol. Med.* (1984) 173:194-199) disclose a human osteogenic protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

[0008] Urist et al. (*Proc. Natl. Acad. Sci. USA* (1984) 81:371-375) disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The authors reported that the protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract from bone results in ill-defined and impure preparations.

[0009] European Patent Application Serial No. 148,155, published October 7, 1985, purports to disclose osteogenic proteins derived from bovine, porcine, and human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into animals.

[0010] International Application No. PCT/087/01537, published January 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative bone inductive factors produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and apparently expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenic proteins, bone induction was not demonstrated. See also Urist et al., EP 0,212,474 entitled Bone Morphogenic Agents.

[0011] Wang et al. (*Proc. Nat. Acad. Sci. USA* (1988) 85: 9484-9488) discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a molecular weight of 30 kD determined from gel elution. Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

[0012] Wozney et al. (*Science* (1988) 242: 1528-1534) discloses the isolation of full-length cDNA's encoding the human equivalents of three polypeptides originally purified from bovine bone. The authors report that each of the three recombinantly expressed human proteins are independently or in combination capable of inducing cartilage formation. No evidence of bone formation is reported.

[0013] It is an object of this invention to provide osteogenic devices comprising matrices containing dispersed osteogenic protein capable of bone induction in allogenic and xenogenic implants. Another object is to provide synthetic osteogenic proteins capable of inducing endochondral bone formation in mammals, including humans. Yet another

object is to provide genes encoding non-native osteogenic proteins and methods for their production using recombinant DNA techniques. Another object is to provide novel biosynthetic forms of osteogenic proteins and a structural design for novel, functional osteogenic proteins. Another object is to provide methods for inducing cartilage formation.

[0014] These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

Summary of the Invention

[0015] This invention involves osteogenic devices which, when implanted in a mammalian body, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation. Suitably modified as disclosed herein, the devices also may be used to induce cartilage formation. The devices comprise a carrier material, referred to herein as a matrix, having the characteristics disclosed below, containing dispersed osteogenic protein in the form of a biosynthetic construct.

[0016] Key to these developments was the successful preparation of substantially pure osteogenic protein by purification from bone, the elucidation of amino acid sequence and structure data of the native osteogenic protein, and insights involving study of the DNA and amino acid sequences of the natural source product. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from mammalian bone. Investigation of the properties and structure of the native form osteogenic protein then permitted the inventors to develop a rational design for non-native forms, i.e., forms never before known in nature, capable of inducing bone formation. As far as applicants are aware, the constructs disclosed herein constitute the first instance of the design of a functional, active protein without preexisting knowledge of the active region of a native form nucleotide or amino acid sequence.

[0017] A series of consensus DNA sequences were designed with the goal of producing an active osteogenic protein. The sequences were based on partial amino acid sequence data obtained from the naturally sourced product and on observed homologies with unrelated genes reported in the literature, or the sequences they encode, having a presumed or demonstrated developmental function. Several of the biosynthetic consensus sequences have been expressed as fusion proteins in prokaryotes, purified, cleaved, refolded, combined with a matrix, implanted in an established animal model, and shown to have endochondral bone-inducing activity. The currently preferred active proteins comprise sequences designated COP5, COP7, COP16, and OP1. The amino acid sequences of these proteins are set forth below.

30

COP5	1 10 20 30 40 LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD 50 60 70 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR
COP7	1 10 20 30 40 LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD 50 60 70 HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR
COP16	1 10 20 30 40 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD 50 60 70 -10 PKHHSQRARKKNKN HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR

55

-5
HQRQA

1 10 20 30 40
OP1 CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
5 50 60 70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
80 90 100
ISVLYFDDSSNVILKKYRNMVVRACGCH

10

[0018] In these sequences and all other amino acid sequences disclosed herein, the dashes (-) are used as fillers only to line up comparable sequences in related proteins, and have no other function. Thus, amino acids 45-50 of COP7, for example, are NHAVV. Also, the numbering of amino acids is selected solely for purposes of facilitating comparisons between sequences. Thus, for example, the DF residues numbered at 9 and 10 of COPS and COP7 may 15 comprise residues, e.g., 35 and 36, of an osteogenic protein embodying the invention. Various leader or trailer sequences may be attached to the operative active region provided the osteogenic or chondrogenic activity of the protein is not destroyed.

[0019] Thus, in one aspect, the invention comprises a protein comprising an amino acid sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that it is capable of inducing endochondral bone formation 20 when properly folded and implanted in a mammal in association with a matrix. Some of these sequences induce cartilage, but not bone. Also, the bone forming materials may be used to produce cartilage if implanted in an avascular locus, or if an inhibitor to full bone development is implanted together with the active protein. Thus, in another aspect, the invention comprises a protein less than about 200 amino acids long (for each chain) including a sequence sufficiently 25 duplicative of the sequence of COP5, COP7, COP16, or OP1 such that it is capable at least of cartilage formation when properly folded and implanted in a mammal in association with a matrix. The phrase "sufficiently duplicative", as used herein, is used to describe proteins having a degree of homology with the specific sequences disclosed herein and other, different amino acids but which nevertheless exhibit osteogenic or chondrogenic activity.

[0020] In one preferred aspect, these proteins comprise species of the generic amino acid sequences:

30

1 10 20 30 40 50
LXVXFDXGWXXXWXXXPXXGXXAXYCXGXCXXPXXXXXXNHAXX
60 70 80 90 100
35 QXXVXXXXNXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

or

40

1 10 20 30 40 50
CXXXXLXVXFDXGWXXXWXXXPXXGXXAXYCXGXCXXPXXXXXXNHAXX
60 70 80 90 100
QXXVXXXXNXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

45

where the letters indicate the amino acid residues of standard single letter code, and the Xs each represent any one of the 22 naturally occurring amino acid residues. Preferred amino acid sequences within the foregoing generic sequences are:

50

1 10 20 30 40 50
 LYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
 5 K S S L QE VIS E FD Y E A AY MPESMKAS VI
 F E K I DN L N S Q ITK F P TL
 A S K
 60 70 80 90 100
 QTLVNSVNPKGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 10 RF T S K DPV V Y N S H RN RS
 N S K P E

and

15 1 10 20 30 40 50
 CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
 RRRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
 20 KE F E K I DN L N S Q ITK F P TL
 Q A S K
 60 70 80 90 100
 QTLVNSVNPKGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 25 RF T S K DPV V Y N S H RN RS
 N S K P E

30 wherein each of the amino acids arranged vertically at each position in the sequence may be used alternatively in various combinations. Note that these generic sequences have 6 and preferably 7 cysteine residues where inter- or
 35 intramolecular disulfide bonds can form, and contain other critical amino acids which influence the tertiary structure of the proteins. These generic structural features are found in previously published sequences, none of which have been described as capable of osteogenic activity, and most of which never have been linked with such activity.

[0021] Particular useful sequences include:

35 Vgl 1 10 20 30 40
 CKKRHLYVEFK-DVGWQNWVIAPIQGYMANCYGECPPYPLTE
 40 50 60 70
 ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMS
 80 90 100
 ISMLFYDNNNDNVVLRHYENMAVDECGCR

45 DPP 1 10 20 30 40
 CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFPLAD
 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 VAMILYLNQSTVVLKNYQEMTVVVGCGCR

50 CBMP-2a 1 10 20 30 40
 CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
 80 90 100
 ISMLYLDENEKVVVLKNYQDMVVEGCGCR

5	CBMP-2b 1 10 20 30 40 CRRHSLYVDFS-DVGWNDWIVAPPQAFYCHGDCPFPLAD 50 60 70 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA 80 90 100 ISMLYLDEYDKVVLKNYQEMVVEGCGCR
10	CBMP-3 1 10 20 30 40 CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK 50 60 70 SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEK MSS 80 90 100 LSILFFDENKNVVLKVNPNMTVESCACR
15	COP1 1 10 20 30 40 LYVDFQRDVGGWDDWIIAPVDFDAYYCSGACQFPSAD 50 60 70 HFNSTN--H-AVVQTLVNNMNP GK-VPKPCCVPTELSA 80 90 100 ISMLYLDENSTVVLKNYQEMTVVVGCGCR
20	COP3 1 10 20 30 40 LYVDFQRDVGGWDDWIVAPPQAFYCSGACQFPSAD 50 60 70 HFNSTN--H-AVVQTLVNNMNP GK-VPKPCCVPTELSA 80 90 100 ISMLYLDENEKVV LKNYQEMVVEGCGCR
25	COP4 1 10 20 30 40 LYVDFQRDVGGWDDWIVAPPQAFYCSGACQFPSAD 50 60 70 HFNSTN--H-AVVQTLVNNMNP GK-VPKPCCVPTELSA 80 90 100 ISMLYLDENEKVV LKNYQEMVVEGCGCR
30	COP5 1 10 20 30 40 LYVDFQRDVGGWDDWIVAPPQAFYCSGACQFPSAD 50 60 70 HFNSTN--H-AVVQTLVNNMNP GK-VPKPCCVPTELSA 80 90 100 ISMLYLDENEKVV LKNYQEMVVEGCGCR
35	COP7 1 10 20 30 40 LYVDFQRDVGGWDDWIVAPPQAFYCSGACQFPSAD 50 60 70 HFNSTN--H-AVVQTLVNNMNP GK-VPKPCCVPTELSA 80 90 100 ISMLYLDENEKVV LKNYQEMVVEGCGCR

40 Vg1 is a known *Xenopus* sequence heretofore not associated with bone formation. DPP is an amino acid sequence encoded by a *Drosophila* gene responsible for development of the dorsoventral pattern. OP1 is a region of a natural sequence encoded by exons of a genomic DNA sequence retrieved by applicants. The CBMPs are amino acid sequences comprising subparts of mammalian proteins encoded by genomic DNAs and cDNAs retrieved by applicants. The COPs are totally biosynthetic protein sequences expressed by novel consensus gene constructs, designed using the criteria set forth herein, and not yet found in nature.

45 [0022] These proteins are believed to be dimers. They appear not to be active when reduced. Various combinations of species of the proteins may exist as heterodimers or homodimers. As far as applicants are aware, the COP5, COP7, COP16, and OP1 constructs constitute the first instances of the design of a bioactive protein without preexisting knowledge of the active region of a native form nucleotide or amino acid sequence.

50 [0023] The invention thus provides synthetic osteogenic protein produced using recombinant DNA techniques. The protein may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native protein, no matter how derived. In view of this disclosure, skilled genetic engineers can design and synthesize genes which encode appropriate amino acid sequences, and then can express them in various types of host cells, including both prokaryotes and eucaryotes, to produce large quantities of active synthetic proteins comprising truncated analogs, muteins, fusion proteins, and other constructs mimicking the biological activity of the native forms and capable of inducing bone formation in mammals including humans.

55 [0024] The synthetic proteins are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles or porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range

of 70 - 850 μ m, preferably 70 - 420 μ m. It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable *in vivo* to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and particulate, deglycoslated (or HF treated), protein extracted, demineralized, xenogenic bone. Optionally, such xenogenic bone powder matrices also may be treated with proteases such as trypsin. Other useful matrix materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates.

[0025] The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1:959-963). The devices may be used to induce local endochondral bone formation in non-union fractures, and in other clinical applications including periodontal applications where bone formation is required. Another potential clinical application is in cartilage repair, for example, in the treatment of osteoarthritis.

Brief Description of the Drawing

[0026] The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1 is a comparison of the amino acid sequence of various osteogenic proteins to those of the TGF-beta family. COP1, COP3, COP4, COP5, and COP7 are a family of analogs of synthetic osteogenic proteins developed from the consensus gene that was joined to a leader protein via a hinge region having the sequence D-P-N-G that permitted chemical cleavage at the D-P site (by acid) or N-G (by hydroxylamine) resulting in the release of the analog protein; VG1 is a *Xenopus* protein, DPP is a *Drosophila* protein; OP1 is a native osteogenic protein; CBMP2a and 2b, and CBMP3 are subparts of proteins disclosed in PCT application 087/01537; MIS is Mullerian inhibitory substance; and "consensus choices" represent various substitutions of amino acids that may be made at various positions in osteogenic proteins;

FIGURE 2A is an *E. coli* expression vector containing a gene of an osteogenic protein fused to a leader protein;

FIGURE 2B is the DNA sequence comprising a modified trp-LE leader, two Fb domains of protein A, an ASP-PRO cleavage site, and the COP5 sequence;

FIGURES 3A and 3B are photomicrographs of implants showing the histology (day 12) of COP5 active recombinant protein. A is a control (rat matrix alone, 25 mg). B is rat matrix plus COP5, showing +++ cartilage formation and ++ bone formation (see key infra). Similar results are achieved with COP7; and

FIGURE 4 is a schematic representation of the DNA sequence and corresponding amino acid sequence of a consensus gene for osteogenic protein (COPO).

Description

[0027] Purification protocols have been developed which enable isolation of the osteogenic protein present in crude protein extracts from mammalian bone. The isolation procedure enables the production of significant quantities of substantially pure osteogenic protein from any mammalian species, provided sufficient amounts of fresh bone from the species is available. The empirical development of the procedure, coupled with the availability of fresh calf bone, enabled isolation of substantially pure bovine osteogenic protein (BOP). BOP has been characterized significantly; its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat have been studied; it has been shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in heterogeneous bone extracts; and it may be used to induce formation of endochondral bone in orthopedic defects including non-union fractures. In its native form it is a glycosylated, dimeric protein. However, it is active in deglycosylated form. It has been partially sequenced.

[0028] Elucidation of the amino acid sequence of BOP enabled the construction of a consensus nucleic acid sequence designed as disclosed herein based on the sequence data, inferred codons for the sequences, and observation of partial homology with known genes.

[0029] These consensus sequences have been refined by comparison with the sequences present in certain regu-

latory genes from *drosophila*, *xenopus*, and human followed by point mutation, expression, and assay for activity. This approach has been successful in producing several active totally synthetic constructs not found in nature (as far as applicants are aware) which have true osteogenic activity.

5 [0030] These discoveries enable the construction of DNAs encoding totally novel, non-native protein constructs which individually and combined are capable of producing true endochondral bone. The DNAs may be expressed using well established recombinant DNA technologies in prokaryotic or eucaryotic host cells, and the expressed proteins may be oxidized and refolded *in vitro* if necessary for biological activity.

10 [0031] The design and production of such biosynthetic proteins, the nature of the matrix, and other material aspects concerning the nature, utility, how to make, and how to use the subject matter claimed herein will be further understood from the following, which constitutes the best method currently known for practicing the various aspects of the invention.

CONSENSUS SEQUENCE DESIGN

15 [0032] A synthetic consensus gene shown in FIGURE 4 was designed to encode a consensus protein based on amino acid predictions from homology with the TGF-beta gene family. The designed consensus sequence was then constructed using known techniques involving assembly of oligonucleotides manufactured in a DNA synthesizer.

20 [0033] Tryptic peptides derived from Bovine Osteogenic Protein isolated by applicants and sequenced by Edman degradation provided amino acid sequences that showed strong homology with the Drosophila DPP protein sequence (as inferred from the gene), the Xenopus VG1 protein, and somewhat less homology to inhibin and TGF-beta, as demonstrated below in TABLE 1.

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TABLE 1

<u>protein</u>	<u>amino acid sequence</u>	<u>homology</u>
(<u>BOP</u>)	SFDAYYCSGACQFPS ***** * * *	(9/15 matches)
(<u>DPP</u>)	GYDAYYCHGKCPFFL	
(<u>BOP</u>)	SFDAYYCSGACQFPS * * * * *	(6/15 matches)
(<u>Vgl</u>)	GYMANCYGECPYPL	
(<u>BOP</u>)	SFDAYYCSGACQFPS * * * * *	(5/15 matches)
(<u>inhibin</u>)	GYHANYCEGECPSHI	
(<u>BOP</u>)	SFDAYYCSGACQFPS * * * *	(4/15 matches)
(<u>TGF-beta</u>)	GYHANFCLGPCPYIW	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDEN ***** * **** * *	(12/20 matches)
(<u>Vgl</u>)	LPCCVPTKMSPIISMLFYDNN	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDEN * ***** * **** *	(12/20 matches)
(<u>inhibin</u>)	KSCCVPTKLRPMMSMLYYDDG	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDE **** * * *	(6/19 matches)
(<u>TGF-beta</u>)	APCCVPQALEPLPIVYYVG	
(<u>BOP</u>)	K/RACCVPTELSAISMLYLDEN ***** * ****	(12/20 matches)
(<u>DPP</u>)	KACCVPTQLDSVAMLYLNDQ	

5	(<u>BOP</u>)	LYVDF *****	(5/5 matches)
	(<u>DPP</u>)	LYVDF	
10	(<u>BOP</u>)	LYVDF *** * *	(4/5 matches)
	(<u>Vg1</u>)	LYVEF	
15	(<u>BOP</u>)	LYVDF ** **	(4/5 matches)
	(<u>TGF-beta</u>)	LYIDF	
20	(<u>BOP</u>)	LYVDF * *	(2/5 matches)
	(<u>inhibin</u>)	FFVSF	
25	<u>*-match</u>		

[0034] In determining an appropriate amino acid sequence of an osteogenic protein (from which the nucleic acid sequence can be determined), the following points were considered: (1) the amino acid sequence determined by Edman degradation of natural source osteogenic protein tryptic fragments is ranked -highest as long as it has a strong signal and shows homology or conservative changes when aligned with the other members of the gene family; (2) where the sequence matches for all four proteins, it is used in the synthetic gene sequence; (3) matching amino acids in DPP and Vg1 are used; (4) If Vg1 or DPP diverged but either one were matched by inhibin or by TGF-beta, this matched amino acid is chosen; (5) where all sequences diverged, the DPP sequence is initially chosen, with a later plan of creating the Vg1 sequence by mutagenesis kept as a possibility. In addition, the consensus sequence is designed to preserve the disulfide crosslinking and the apparent structural homology among the related proteins.

RECOMBINANT OSTEOGENIC PROTEIN CONSTRUCTS

[0035] This approach resulted in the production of novel recombinant proteins capable of inducing formation of cartilage and endochondral bone comprising a protein structure analogous to or duplicative of the functional domain of the naturally sourced material. The amino acid sequences encoded by the consensus DNA sequences were derived from a family of natural proteins implicated in tissue development. These gene products/proteins are known to exist in active form as dimers and are, in general, processed from a precursor protein to produce an active C-terminal domain of the precursor.

[0036] The recombinant osteogenic/chondrogenic proteins are "novel" in the sense that, as far as applicants are aware, they do not exist in nature or, if they do exist, have never before been associated with bone or cartilage formation. The approach to design of these proteins is to employ amino acid sequences, found in the native OP isolates, in polypeptide structures are patterned after certain proteins reported in the literature, or the amino acid sequences inferred from DNAs reported in the literature. Thus, using the design criteria set forth above, and refining the amino acid sequence as more protein sequence information was learned, a series of synthetic proteins were designed with the hope and intent that they might have osteogenic or chondrogenic activity when tested in the bioassay system disclosed below.

[0037] It was noted, for example, that DPP from *drosophila*, VG1 from *Xenopus*, the TGF beta family of proteins, and to a lesser extent, alpha and beta inhibins, had significant homologies with certain of the sequences derived from the naturally sourced OP product. (FIGURE 1.) Study of these proteins led to the realization that a portion of the sequence of each had a structural similarity observable by analysis of the positional relationship of cysteines and other amino acids which have an important influence on three dimensional protein conformation. It was noted that a region

of these sequences had a series of seven cysteines, placed very nearly in the same relative positions, and certain other amino acids in sequence as set forth below:

5
10 20 30 40 50
CXXXXLXVXFDXGWWXXXPXXGXXAXYCXGXCXXPXXXXXXNHA~~XX~~
60 70 80 90 100
QXXVXXXXNXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

10
wherein each X independently represents an amino acid. Expression experiments of two of these constructs demonstrate activity. Expression experiments with constructs patterned after this template amino acid sequence with a shorter sequence having only six cysteines also show activity:

15
10 20 30 40 50
LXVXFDXGWWXXXPXXGXXAXYCXGXCXXPXXXXXXNHA~~XX~~
60 70 80 90 100
QXXVXXXXNXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

20
wherein each X independently represents an amino acid. Within these generic structures are a multiplicity of specific sequences which have osteogenic or chondrogenic activity. Preferred structures are those having the amino acid sequence:

25
10 20 30 40 50
CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
RKRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
KE F E K I DN L N S Q ITK F P TL
30 35 40 45 50
Q A S K
60 70 80 90 100
QTLVNSVNP~~G~~KIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR
SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
RF T S K DPV V Y N S H RN RS
35 40 45 50 55
N S K P E

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be used. Novel active proteins also are defined by amino acid sequences comprising an active domain beginning at residue number 6 of this sequence, i.e. omitting the N terminal CXXXX, or omitting any of the preferred specific combinations such as CKRHP, CRRKQ, CKRHE, etc, resulting in a construct having only six cysteine residues. After this work, PCT 40
87/01537 was published, and it was observed that the proteins there identified as BMPII a and b and BMPIII each included a region embodying this generic structure. These proteins were not demonstrated to be osteogenic in the published application. However, applicants discovered that a subpart of the amino acid sequence of these proteins, properly folded, and implanted as set forth herein, is active. These are disclosed herein as CBMPIIa, CBMPIIb, and 45
CBMPIII. Also, applicants retrieved a previously unreported gene by probing a human genomic DNA library with COPO. This protein was designated OP1. It comprises a region exhibiting the same generic structure.

[0038] Thus, the preferred osteogenic proteins are expressed from recombinant DNA and comprise amino acid sequences including any of the following sequences:

5
 Vgl 1 10 20 30 40
 CKKRHLYVEFK-DVGWQNWWIAPQGYMANCYGECPYPLTE
 50 60 70
 ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
 80 90 100
 ISMLFYDNNNDNVVLRHYENMAVDECGCR

10 DPP 1 10 20 30 40
 CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFPLAD
 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 VAMLYLNDQSTVVLKNYQEMTVVGCGR

15
 OPI 1 10 20 30 40
 LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
 50 60 70
 20 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

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-5
HQRQA

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OP1 1 10 20 30 40
CKKHELYVSFR-DLGWQDWII APEGYAAYYCEGECAFPLNS
50 60 70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPQLNA
80 90 100
ISVLYFDDSSNVILKKYRNMVVRACGCH

10

CBMP-2a 1 10 20 30 40
CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
50 60 70
HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
80 90 100
ISMLYLDENEKVVALKNYQDMVVEGCGCR

15

CBMP-2b 1 10 20 30 40
CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
50 60 70
HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
80 90 100
ISMLYLDEYDKVVALKNYQEMVVEGCGCR

20

25

CBMP-3 1 10 20 30 40
CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
50 60 70
SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
80 90 100
LSILFFDENKNVVLKVYPNMTVESACR

30

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COP1 1 10 20 30 40
LYVDFQRDVGVDDWIIAPVDFDAYYCSGACQFPSAD
50 60 70
HFNSTN--H-AVVQTLVNNMNPKG-VPKPCCVPTELSA
80 90 100
ISMLYLDENSTVVLKNYQEMTVVGCGR

40

45

COP3 1 10 20 30 40
LYVDFQRDVGVDDWIVAPPGYQAFYCSGACQFPSAD
50 60 70
HFNSTN--H-AVVQTLVNNMNPKG-VPKPCCVPTELSA
80 90 100
ISMLYLDENEKVVALKNYQEMVVEGCGCR

50

55

5	COP4 1 10 20 30 40 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD 50 60 70 HFNSTN--H-AVVQTLVNNMNPKG-VPKPCCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR
10	COP5 1 10 20 30 40 LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD 50 60 70 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR
15	COP7 1 10 20 30 40 LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD 50 60 70 HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR
20	COP16 1 10 20 30 40 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD 50 60 70 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR
25	1 10 20 30 40 PKHHSQRARKKNKN CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD 50 60 70 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA 80 90 100 ISMLYLDENEKVLKNYQEMVVEGCGCR
30	

[0039] As shown in FIGURE 1, these sequences have considerable homology with the alpha and beta inhibins, three forms of TGF beta, and MIS.

35

Gene Preparation

[0040] The synthetic genes designed to express the proteins as described above preferably are produced by assembly of chemically synthesized oligonucleotides. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer (TBE). The DNA is then electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purified by PAGE. Natural gene sequences and cDNAs also may be used for expression.

45

Expression

[0041] The genes can be expressed in appropriate prokaryotic hosts such as various strains of *E. coli* and also in bacillus, yeasts, and various animal cells such as CHO, myeloma, etc. Generally, expression may be achieved using many cell types and expression systems well known to those skilled in the art. If the gene is to be expressed in *E. coli*, it must first be cloned into an expression vector. An expression vector (FIGURE 2A) based on pBR322 and containing a synthetic trp promoter operator and the modified trp LE leader can be opened at the EcoRI and PstI restriction sites, and a FB-FB COP1, COP3, COP5, and COP7 gene fragments (FIGURE 2B) can be inserted between these sites, where FB is fragment B of Staphylococcal Protein A. The expressed fusion protein results from attachment of the COP gene to a fragment encoding FB. The COP protein is joined to the leader protein via a hinge region having the sequence Asp-Pro-Asn-Gly. This hinge permits chemical cleavage of the fusion protein with dilute acid at the asp-pro site or cleavage at Asn-Gly with hydroxylamine, resulting in release of the COP protein. For COP16 and OP1, the proteins are expressed as fusion products, using the modified trp-LE as a leader.

Production of Active Proteins

[0042] The following procedure was followed for production of active recombinant proteins. *E. coli* cells containing the fusion proteins were lysed. The fusion proteins were purified by differential solubilization. In the case of the COP1, 3, 4, 5, and 7 fusion proteins, cleavage was with dilute acid, and the resulting cleavage products were passed through a Sephadryl-200HR column. The Sephadryl column separated most of the uncleaved fusion products from the COP1, 3, 4, 5, and 7 analogs. In the case of the COP16 or OP1 fusion protein, cleavage was with a more concentrated acid, and an SP-Trisacryl column was used as an additional purification step. The COP or OP fractions were then subjected to HPLC on a semi-prep C-18 column.

[0043] Initial conditions for refolding of COP analogs or OP1 were at pH 8.0 using Tris, Gu-HCl, dithiothreitol. Final conditions for refolding of COP analogs were at pH 8.0 using Tris, oxidized glutathione, and lower amounts of Gu-HCl and dithiothreitol. Alternatively, the COP or OP1 proteins are suspended in 50 mM HCl, 6 M guanidine-HCl, pH 8.0, for 18 hours at 4°C. Refolding may not be required if the proteins are expressed in animal cells.

15 Production of Antisera

[0044] Antisera to COP7 and COP5 were produced in New Zealand white rabbits. Western blots demonstrate that the antisera react with COP7 and COP5 preparations. Antisera to COP7 has been tested for reactivity to naturally sourced bovine osteogenic protein samples. Western blots show a clear reaction with the 30 kD protein and, when reduced, with the 16 kD subunit. The immunoreactive species appears as a closely-spaced doublet in the 16 kD subunit region, similar to the 16 kD doublet seen in Con A blots.

MATRIX PREPARATION

25 General Consideration of Matrix Properties

[0045] The carrier described in the bioassay section, infra, may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., HAP, collagen, tricalcium phosphate, or polylactic acid, polyglycolic acid and various copolymers thereof). Also xenogeneic bone may be used if pretreated as described below.

[0046] Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone; particles between 75 and 420 μ m elicit the maximum response. Contamination of the matrix with bone mineral will inhibit bone formation. Most importantly, the procedures used to formulate osteogenic protein onto the matrix are extremely sensitive to the physical and chemical state of both the osteogenic protein and the matrix.

[0047] The sequential cellular reactions at the interface of the bone matrix/OP implants are complex. The multistep cascade includes: binding of fibrin and fibronectin to implanted matrix, chemotaxis of cells, proliferation of fibroblasts, differentiation into chondroblasts, cartilage formation, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

[0048] A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible *in vivo* and biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Biocompatibility requires that the matrix not induce significant inflammation when implanted and not be rejected by the host animal. Biodegradability requires that the matrix be slowly absorbed by the body of the host during development of new bone or cartilage. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates *in vivo*. In bones, the dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

[0049] Matrix geometry, particle size, the presence of surface charge, and porosity or the presence of interstices among the particles of a size sufficient to permit cell infiltration, are all important to successful matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

[0050] The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue. Masticated muscle or other tissue may also be used. Large allogeneic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particles and the dispersed osteogenic protein.

Preparation of Biologically Active Allogenic Matrix

5 [0051] Demineralized bone matrix is prepared from the dehydrated diaphyseal shafts of rat femur and tibia as described herein to produce a bone particle size which pass through a 420 μm sieve. The bone particles are subjected to dissociative extraction with 4 M guanidine-HCl. Such treatment results in a complete loss of the inherent ability of the bone matrix to induce endochondral bone differentiation. The remaining insoluble material is used to fabricate the matrix. The material is mostly collagenous in nature, and upon implantation, does not induce cartilage and bone. All new preparations are tested for mineral content and false positives before use. The total loss of biological activity of bone matrix is restored when an active osteoinductive protein fraction or a pure protein is reconstituted with the biologically inactive insoluble collagenous matrix. The osteoinductive protein can be obtained from any vertebrate, e.g., bovine, porcine, monkey, or human, or produced using recombinant DNA techniques.

Preparation of Deglycosylated Bone Matrix for Use in Xenogenic Implant

10 15 [0052] When osteogenic protein is reconstituted with collagenous bone matrix from other species and implanted in rat, no bone is formed. This suggests that while the osteogenic protein is xenogenic (not species specific), while the matrix is species specific and cannot be implanted cross species perhaps due to intrinsic immunogenic or inhibitory components. Thus, heretofore, for bone-based matrices, in order for the osteogenic protein to exhibit its full bone inducing activity, a species specific collagenous bone matrix was required.

20 25 [0053] The major component of all bone matrices is Type I collagen. In addition to collagen, extracted bone includes non-collagenous proteins which may account for 5% of its mass. Many non-collagenous components of bone matrix are glycoproteins. Although the biological significance of the glycoproteins in bone formation is not known, they may present themselves as potent antigens by virtue of their carbohydrate content and may constitute immunogenic and/or inhibitory components that are present in xenogenic matrix.

30 35 [0054] It has now been discovered that a collagenous bone matrix may be used as a carrier to effect bone inducing activity in xenogenic implants, if one first removes the immunogenic and inhibitory components from the matrix. The matrix is deglycosylated chemically using, for example, hydrogen fluoride to achieve this purpose.

[0055] Bovine bone residue prepared as described above is sieved, and particles of the 74-420 μm are collected. The sample is dried in vacuo over P_2O_5 , transferred to the reaction vessel and anhydrous hydrogen fluoride (HF) (10-20 ml/g of matrix) is then distilled onto the sample at -70°C. The vessel is allowed to warm to 0°C, and the reaction mixture is stirred at this temperature for 120 min. After evaporation of the HF in vacuo, the residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid.

[0056] Extent of deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with HF, after washing the samples appropriately to remove non-covalently bound carbohydrates.

40 45 [0057] The deglycosylated bone matrix is next treated as set forth below:

1) suspend in TBS (Tris-buffered Saline) 1g/200 ml and stir at 4°C for 2 hrs; or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS) and stir at RT for 30 min;

2) centrifuge and wash with TBS or UTBS as in step 1; and

3) centrifuge; discard supernatant; water wash residue; and then lyophilize.

FABRICATION OF DEVICE

45 [0058] Fabrication of osteogenic devices using any of the matrices set forth above with any of the osteogenic proteins described above may be performed as follows.

A. Ethanol precipitation

50 [0059] In this procedure, matrix is added to osteogenic protein in guanidine-HCl. Samples are vortexed and incubated at a low temperature. Samples are then further vortexed. Cold absolute ethanol is added to the mixture which is then stirred and incubated. After centrifugation (microfuge high speed) the supernatant is discarded. The reconstituted matrix is washed with cold concentrated ethanol in water and then lyophilized.

B. Acetonitrile Trifluoroacetic Acid Lyophilization

[0060] In this procedure, osteogenic protein in an acetonitrile trifluoroacetic acid (ACN/TFA) solution is added to the

carrier. Samples are vigorously vortexed many times and then lyophilized.

C. Urea Lyophilization

5 [0061] For those proteins that are prepared in urea buffer, the protein is mixed with the matrix, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

IN VIVO RAT BIOASSAY

10 [0062] Several of the synthetic proteins have been incorporated in matrices to produce osteogenic devices, and assayed in rat for endochondral bone. Studies in rats show the osteogenic effect to be dependent on the dose of osteogenic protein dispersed in the osteogenic device. No activity is observed if the matrix is implanted alone. The following sets forth guidelines for how the osteogenic devices disclosed herein can be assayed for evaluating protein constructs and matrices for biological activity.

15

A. Subcutaneous Implantation

20 [0063] The bioassay for bone induction as described by Sampath and Reddi (Proc. Natl. Acad. Sci. USA (1983) 80: 6591-6595), herein incorporated by reference, is used to assess endochondral bone differentiation activity. This assay consists of implanting the test samples in subcutaneous sites in allogenic recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants were removed on day 12. The 25 heterotopic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotopic sites.

B. Cellular Events

30 [0064] The implant model in rats exhibits a controlled progression through the stages of matrix induced endochondral bone development including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

C. Histological Evaluation

40 [0065] Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in parafilm, cut into 6-8 mm sections. Staining with toluidine blue or hematoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve day implants are usually sufficient to determine whether the implants show bone inducing activity.

45 D. Biological Markers

50 [0066] Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology should have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and obtaining an estimate of bone formation very quickly after the implants are removed from the rat. Alternatively the amount of bone formation can be determined by measuring the calcium content of the implant.

55 [0067] The osteogenic activity due to osteogenic protein is represented by "bone forming units". One bone forming unit represents the amount of protein that is needed for half maximal bone forming activity as compared to rat demineralized bone matrix as control and determined by calcium content of the implant on day 12.

[0068] Devices that contained only rat carrier show complete absence of new bone formation. The implant consists of carrier rat matrix and surrounding mesenchymal cells. Again, the devices that contained rat carrier and not correctly folded (or biologically inactive) recombinant protein also showed complete absence of bone formation. These implants

are scored as cartilage formation (-) and bone formation (-). The endochondral bone formation activity is scored as zero percent (0%) (FIGURE 3A).

[0069] Implants included biologically active recombinant protein, however, showed evidence of endochondral bone formation. Histologically they showed new cartilage and bone formation.

5 [0070] The cartilage formation is scored as (+) by the presence of metachromatically stained chondrocytes in the center of the implant, as (++) by the presence of numerous chondrocytes in many areas of the implant and as (+++) by the presence of abundant chondrocytes forming cartilage matrix and the appearance of hypertrophied chondrocytes accompanying cartilage calcification (FIGURE 3B).

10 [0071] The bone formation is scored as (+) by the presence of osteoblast surrounding vascular endothelium forming new matrix, as (++) by the formation of bone due to osteoblasts (as indicated by arrows) and further bone remodeling by the appearance of osteoclasts in opposition to the newly formed bone matrix. Vascular invasion is evident in these implants (FIGURE 3B). Formation is scored as (+++) by the presence of extensive remodeled bone which results in the formation of ossicles.

15 [0072] The overall bone inducing activity due to recombinant protein is represented as percent response of endochondral bone formation (see TABLE 2 below).

TABLE 2

HISTOLOGICAL EVALUATION OF RECOMBINANT BONE INDUCTIVE PROTEINS				
	Sample No.	Implanted Protein	Cartilage Formation	Bone Formation
20	260-54	COP-5	+++	++
	279-5	COP-5	++	+
25	285-13	COP-5	+++	++
	277-7	COP-7	+++	++
	277-8	COP-7	+++	++
	277-9	COP-7	++	+
30	285-14	COP-7	+++	++
	285-24	COP-7	++	+
	285-25	COP-7	++	++
35	314-6	COP-16	+++	+++
	314-15	COP-16	++	+
	314-16	COP-16	++	+
	314-12	OP-1	++	+

[0073] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

40 [0074] The invention relates to an osteogenic device for implantation in a mammal, said device comprising a biocompatible, *in vivo* biodegradable matrix defining pores of a dimension sufficient to permit influx, proliferation and differentiation of migratory progenitor cells from the body of said mammal; and a protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains, each of which comprises an amino acid sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing endochondral bone formation in association with said matrix when implanted in a mammal.

45 [0075] Also covered by the invention is a device for implantation in a mammal, said device comprising a biocompatible, *in vivo* biodegradable matrix defining pores of a dimension sufficient to permit influx, proliferation and differentiation of migratory progenitor cells from the body of said mammal; and a protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains, each of which has less than about 200 amino acids, and each of which comprises a sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing cartilage formation in association with said matrix when implanted in a mammal.

50 [0076] The sequence may comprise

10 20 30 40 50
 CXXXXLXVXFDXGWXXWXXXPGXXAXYCXGXCXXPXXXXXXNHAXX
 5 60 70 80 90 100
 QXXVXXXNXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

10 wherein each X independently represents an amino acid; or

10 20 30 40 50
 LXVXFDXGWXXWXXXPGXXAXYCXGXCXXPXXXXXXNHAXX
 15 60 70 80 90 100
 QXXVXXXNXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

20 wherein each X independently represents an amino acid; or

10 20 30 40 50
 CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
 RKRS K S S L QE VIS E FD Y E A AY MPESMKAS VI
 25 KE F E K I DN L N S Q ITK F P TL
 Q A S K
 60 70 80 90 100
 QTLVNSVNPKGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGGCR
 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 30 RF T S K DPV V Y N S H RN RS
 N S K P E

35 wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position; or

10 20 30 40 50
 LYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV
 40 K S S L QE VIS E FD Y E A AY MPESMKAS VI
 F E K I DN L N S Q ITK F P TL
 A S K
 60 70 80 90 100
 QTLVNSVNPKGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGGCR
 45 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S K DPV V Y N S H RN RS
 N S K P E

50 wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position; or

1 10 20 30 40
Vgl CKKRHLVYEFK-DVGWQNWVIAAPQGYMANCYGECPYPLTE
50 60 70
ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP
80 90 100
ISMLFYDNNNDNVVLRHYENMAVDECGR

10

or

1 10 20 30 40
 DPP CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFPLAD
 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 VAMLYLNDQSTVVLKNYQEMTVVGCGCR

20

or

25

OP1 1 10 20 30 40
LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
50 60 70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
80 90 100
ISVLYFDDSSNVILKKYRNMVVRACGCH

35 or

40 OP1 1 10 - 20 30 HQRQA 40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
50 60 70
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTLNA
80 90 100
45 ISVLYFDDSSNVILKKYRNMVVRACGCH

or

50

CBMP-2a	1	10	20	30	40
	CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD				
	50	60	70		
	HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTESA				
	80	90	100		
	TSMIYI.DENEKVVLKNYODMVVEGCGCR				

or

5 1 10 20 30 40
 CBMP-2b CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
 80 90 100
 10 ISMLYLDEYDKVVLKNYQEMVVEGCGCR

or

15 1 10 20 30 40
 CBMP-3 CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
 50 60 70
 SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMSS
 80 90 100
 20 LSILFFDENKNVVLKVVYPNMTVESACR

25 or
 25

30 1 10 20 30 40
 COP1 LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPKG-VPKPCCVPTELSA
 80 90 100
 35 ISMLYLDENSTVVLKNYQEMTVVVGCGCR

35 or
 40

40 1 10 20 30 40
 COP3 LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPKG-VPKPCCVPTELSA
 80 90 100
 45 ISMLYLDENEKVVVLKNYQEMVVEGCGCR

45 or
 50

50 1 10 20 30 40
 COP4 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPKG-VPKPCCVPTELSA
 80 90 100
 55 ISMLYLDENEKVVVLKNYQEMVVEGCGCR

55 or

1 10 20 30 40
 COP5 LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
 5 50 60 70
 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
 80 90 100
 ISMLYLDENEKVVALKNYQEMVVEGCGCR

10

or

15 1 10 20 30 40
 COP7 LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
 50 60 70
 HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
 80 90 100
 ISMLYLDENEKVVALKNYQEMVVEGCGCR

20

or

25 1 10
 COP16 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD
 50 60 70 80 90 100
 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
 ISMLYLDENEKVVALKNYQEMVVEGCGCR

35 [0077] The device of the invention may comprise an osteogenic protein comprising a pair of separate polypeptide chains.

[0078] The invention also relates to an osteogenic protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains each of which comprises a sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing endochondral bone formation in association with a matrix when implanted in a mammal.

[0079] In another embodiment, the invention relates to a protein, produced by expression of recombinant DNA in a host cell, comprising one or more polypeptide chains less than about 200 amino acids long and comprising a sequence sufficiently duplicative of the sequence of COP5, COP7, COP16, or OP1 such that said protein is capable of inducing cartilage formation in association with a matrix when implanted in a mammal.

40 [0080] The protein may be unglycosylated, and may comprise the amino acid sequences:

50 10 20 30 40 50
 CXXXXLXVXFDXGWXWXXXPGXXAXYCXGXCXXPXXXXXXXXXNHAXX
 60 70 80 90 100
 QXXVXXXNXXXXPXXCCXPXXXXXXXXXXXXVXLXXYXXMXVXXCXCX

55 or

10 20 30 . 40 50
LXVXFDXGWXXWXXXPXGXXAXYCXGXCXXPXXXXXXXXXNHAXX
60 70 80 90 100
0XXVXXXXNXXXXXPXXCCXPXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX

wherein each X independently represents an amino acid.

[0081] The protein of the invention may comprise the amino acid sequences:

10	20	30	40	50	
CKRHPLYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHNLNSTNHAIV					
RKRS K S S L	QE VIS E FD Y	E A AY	MPESMKAS	VI	
KE F E K I	DN	L	N S	Q ITK F P	TL
Q A S	K				
60	70	80	90	100	
QTLVNSVNPCKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCCRC					
SI HAI SEQV EP	A EQMNSLAI	FFNDQDK	I RK EE T DA H H		
RF T S	K DPV V	Y N S	H RN	RS	
N S		K	P	E	

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position.

[0082] The protein of the invention may comprise the amino acid sequences:

10	20	30	40	50	
LYVDFRDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIV					
K S S L	QE VIS E FD Y	E A AY	MPESMKAS	VI	
F E K I	DN	L	N S	Q ITK F P	TL
A S	K				
60	70	80	90	100	
QTLVNSVNPKGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVVEGCGCR					
SI HAI SEQV EP	A EQMNSLAI	FFNDQDK	I RK EE T	DA H H	
RF T	S	K DPV V	Y N S	H RN	RS
N S			K	P	E

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position.

[0083] The protein of the invention may comprise the amino acid sequences:

Vg1	1	10	20	30	40
	CKKRHLYVEFK-DVGWQNWVIAPQGYMANCYGECPYPLTE				
	50	60	70		
	ILNGSN--H-AILQTLVHSIEPED-IPLPCCVPTKMSP				
	80	90	100		
	ISMLFYDNNNDNVVLRHYENMAVDECGR				

or

1 10 20 30 40
 DPP CRRHSLYVDFS-DVGWDDWIVAPLGYDAYYCHGKCPFPLAD
 5 50 60 70
 HFNSTN--H-AVVQTLVNNNNPGK-VPKACCVPTQLDS
 80 90 100
 VAMLYLNDQSTTVLKNYQEMTVVGCGR

10 or

1 10 20 30 40
 15 OPI LYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 20 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

or

25
 30 OPI 1 10 20 30 40
 CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS
 50 60 70
 YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA
 80 90 100
 ISVLYFDDSSNVILKKYRNMVVRACGCH

35

or

40 CBMP-2a 1 10 20 30 40
 CKRHPLYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-K-IPKACCVPTELSA
 45 80 90 100
 ISMLYLDENEKVVLKNQDMVVEGCGCR

or

50 CBMP-2b 1 10 20 30 40
 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGDCPFPLAD
 50 60 70
 HLNSTN--H-AIVQTLVNSVNS-S-IPKACCVPTELSA
 55 80 90 100
 ISMLYLDEYDKVVLKNQEMVVEGCGCR

or

5 CBMP-3 1 10 20 30 40
 CARRYLKVDFA-DIGWSEWIISPKSFDAYYCSGACQFPMPK
 50 60 70
 .. SLKPSN--H-ATIQSIVRAVGVVPGIPEPCCVPEKMS
 80 90 100
 10 LSILFFDENKNVVLKVYPNMTVESACR

10

or

15 COP1 1 10 20 30 40
 LYVDFQRDVGVWDDWIIAPVDFDAYYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPNGK-VPKPCCVPTELSA
 20 80 90 100
 ISMLYLDENSTVVLKNYQEMTVVGCGCR

20

or

25 COP3 1 10 20 30 40
 LYVDFQRDVGVWDDWIVAPPGYQAFYCSGACQFPSAD
 50 60 70
 HFNSTN--H-AVVQTLVNNMNPNGK-VPKPCCVPTELSA
 30 80 90 100
 ISMLYLDENEKVVVLKNYQEMVVEGCGCR

30

or

35 COP4 1 10 20 30 40
 LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD
 40 50 60 70
 HFNSTN--H-AVVQTLVNNMNPNGK-VPKPCCVPTELSA
 80 90 100
 ISMLYLDENEKVVVLKNYQEMVVEGCGCR

40

or

45 COP5 1 10 20 30 40
 LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
 50 60 70
 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
 80 90 100
 55 ISMLYLDENEKVVVLKNYQEMVVEGCGCR

or

1 10 20 30 40
 COP7 LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
 5 50 60 70
 HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
 80 90 100
 ISMLYLDENEKVVALKNYQEMVVEGCGCR

10

or

15 -10
 COP16 PKHHSSRARKKNKN
 1 10 20 30 40
 CRRHSLYVDFS-DVGWNDWIVAPPGYQAFYCHGECPFPLAD
 50 60 70
 HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
 20 80 90 100
 ISMLYLDENEKVVALKNYQEMVVEGCGCR

25 The protein of the invention may comprise the product of expression of a DNA in a prokaryotic cell.
 [0084] Also contemplated by the invention is a cell line engineered to express the protein of the invention.
 [0085] Preferably, the protein has a half maximum bone forming activity of about 20-25 ng per 25 mg of implant.
 [0086] The invention also contemplates antibodies reactive with an epitope of the protein of the invention.
 [0087] The device of the invention preferably includes a matrix comprising demineralized, deglycosylated, protein extracted, particulate, xenogenic bone, or demineralized, protein extracted, particulate, xenogenic bone treated with
 30 HF.

Claims

35 1. Use of a composition for the manufacture of a medicament for inducing bone formation, the composition consisting essentially of osteogenic protein comprising one polypeptide chain encoded by a single DNA sequence as the active osteogenic ingredient, said DNA sequence encoding a polypeptide chain comprising an amino acid sequence sufficiently duplicative of a sequence of any of COP5, COP7, COP16 or OP1 such that said polypeptide chain is capable of inducing cartilage and endochondral bone formation when implanted in a mammal in association with a matrix.

40 2. Osteogenic protein comprising one polypeptide chain capable of inducing endochondral bone formation when disposed within a matrix and implanted in a mammal, wherein the protein comprises a region containing six cysteine residues positioned in the relative positions shown in the sequence:

45

LXXFXDXGWWXXXWPXXGXXAXYCXGXCXXPXXXXXXXXXNHAXXQX
 XVXXXNXXXXPXXCCXPXXXXXXXXXLXXXXXXVXLXXYXXMXVXXCXCX,

50

wherein the letters indicate the amino acid residues of standard single letter code and each X represents any amino acid, with the proviso that dpp is excluded.

55 3. A process for producing active synthetic osteogenic protein as defined in claim 2, the process comprising the steps of: (a) producing a consensus amino acid sequence by comparing amino acid sequences of proteins of the TGF-beta family, which consensus sequence preserves the disulphide crosslinking pattern present in the sequence: (i)

5 LXVXFDXGWWXXXPXXGXXAXYCXGXCXXPXXXXXXXXXNHAXXQX
 XXXXNXXXXPXXCCXPXXXXXXXXXLXXXXXXXXXVLXXYXXMXVXXCXC

10 X; or (ii)

15 CXXXLXVXFDXGWWXXXPXXGXXAXYCXGXCXXPXXXXXXXXXNH
 AXXQXXVXXNXXXXPXXCCXPXXXXXXXXXLXXXXXXXXXVLXXYXXMXV

20 XXCXCX;

wherein the letters indicate the amino acid residues of standard single letter code and each X represents any amino acid; (b) constructing a synthetic gene encoding the consensus amino acid sequence of step (a), for example by assembling chemically synthesised oligonucleotides; (c) introducing the synthetic gene of step (b) into a prokaryotic or eukaryotic host cell; (d) expressing the synthetic gene to produce a putative synthetic osteogenic protein; (e) assaying the putative synthetic osteogenic protein to determine whether it is active in inducing endochondral bone formation when implanted in a mammal, and optionally (f) repeating steps (a) to (e) (or mutagenizing the synthetic gene and repeating steps (c) to (e)) if the putative synthetic osteogenic protein is inactive when so assayed.

25 4. A process for the production of a polypeptide chain for use in the preparation of an active osteogenic protein, comprising the steps of: (a) providing genetic material encoding the osteogenic protein; (b) introducing the genetic material into a prokaryotic host cell; (c) expressing the genetic material in the host cell and (d) refolding the expressed protein by oxidation, wherein the polypeptide chain comprises a region containing six cysteine residues positioned in the relative positions shown in the sequence:

30 LXVXFDXGWWXXXPXXGXXAXYCXGXCXXPXXXXXXXXXNHAXXQX
 XXXXNXXXXPXXCCXPXXXXXXXXXLXXXXXXXXXVLXXYXXMXVXXCXCX,

35 wherein the letters indicate the amino acid residues of standard single letter code and each X represents any amino acid.

40 5. A polypeptide chain producible by the process of claim 4.
 6. The use of claim 1, protein of claim 2, process of claim 3 or claim 4 or polypeptide of claim 5 wherein the protein comprises a region containing seven cysteine residues positioned in the relative positions shown in the sequence:

45 CXXXLXVXFDXGWWXXXPXXGXXAXYCXGXCXXPXXXXXXXXXHA
 XXQXXVXXNXXXXPXXCCXPXXXXXXXXXLXXXXXXXXXVLXXYXXMXV
 XXCXCX.

50 7. The use of claim 1, protein of claim 2, process of claim 3 or claim 4 or polypeptide of claim 5 or use, protein, process or polypeptide of claim 6 wherein the protein is a recombinant protein produced by expression in a host cell.
 8. The process of claim 3 wherein the consensus nucleic acid sequence comprises the sequence: wherein the letters indicate the amino acid residues of standard single letter code and each X represents any amino acid.

(a) LXVXFDXGWWXXXPWXGXXAXYCXGXCXXPXXXXXX
XNHAXXQXXVXXNXXXPXXCCXPXXXXXXLXXXXXX
XVXLXXYXXMXXCXCX; or

(b) CXXXXLXVXFDXGWWWXXXPGXXAXYCXGXCXXPXX
XXXXXXXXNHAXXQXXVXXXNXXXXPXXCCXPXXXXXXLX
XXXXXXXXVXLXXYXXMXVXXCXCX;

15 9. The process of claim 3 or claim 8 wherein the synthetic gene of step (b) comprises a nucleotide sequence which encodes a synthetic polypeptide chain having the same number of cysteine residues in the same relative positions as COP5, COP7, COP16 or OP1.

20 10. The process of any one of claims 3, 8 and 9 wherein the synthetic gene of step (b) comprises a nucleotide sequence which encodes a synthetic polypeptide chain sharing one or more amino acids at corresponding positions in any of the sequences: Vg1, DPP, OP1, CBMP-2a CBMP-2b, CBMP-3, COP1, COP3, COP4, COP5, COP7 or COP16.

11. The process of any one of claims 3 and 8-10 wherein the synthetic gene of step (b) comprises a nucleotide sequence which encodes a synthetic polypeptide chain comprising the amino acid sequence:

(a)

10	20	30	40	50
CKRHPLYVDFRDVGWNDWIVPPGYHAFYCHGECPFPLADHLNSTNHAIV				
RKRS K S S L QE VIS E FD Y E A AY MPESMKAS	VI			
KE F E K I DN L N S Q ITK F P	TL			
Q A S K				
60	70	80	90	100
QTLVNSVNPKGKIPKACCVPTELSAISMLYLDENENVVLKNYQDMVVEGCGCR				
SI. HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H				
RF T S K DPV V Y N S H RN RS				
N S K P E				

wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be in that position; or

(b)

5 10 20 30 40 50
 LYVDFRDVGWNDWIVAPPGYHAFYCHGECPPFPLADHNLNSTNHAIV
 K S S L QE VIS E FD Y E A AY MPESMKAS VI
 F E K I DN L N S Q ITK F P TL
 10 A S K
 60 70 80 90 100
 QTLVNSVNPKGKIPKACCVPTELSAISMLYLDENENVVLKKYQDMVVEGCGCR
 SI HAI SEQV EP A EQMNSLAI FFNDQDK I RK EE T DA H H
 RF T S X DPV V Y N S H RN RS
 15 N S K P E

20 wherein, in each position where more than one amino acid is shown, any one of the amino acids shown may be
 in that position; or

25 (c)
 1 10 20 30 40
 CRRHSLYVDFS - DVGWDDWIVAPLGYDAYYCHGKCPFPLAD
 50 60 70
 HFNSTN -- H - AVVQTLVNNNNPGK - VPKACCVPTQLDS
 30 80 90 100
 VAMLYLNDQSTVVLKNYQEMTVVGCGCR; or

35 (d)
 1 10 20 30 40
 LYVSFR - DLGWQDWIIAPEGYAAYYCEGECAFPLNS
 40 50 60 70
 YMNATN -- H - AIVQTLVHFINPET - VPKPCCAPTQLNA
 45 80 90 100
 ISVLYFDDSSNVILKKYRNRMVVRACGCH; or

(e)

5

-5

HQRQA

1	10	20	30	40
CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNS				
10	50	60	70	
YMNATN--H-AIVQTLVHFINPET-VPKPCCAPTQLNA				
15	80	90	100	
ISVLYFDDSSNVILKKYRNRMVVRACGCH; or				

15

20

1	10	20	30	40
LYVDFQRDVGWDDWIIAPVDFDAYYCSGACQFPSAD				
25	50	60	70	
HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTELSA				
30	80	90	100	
ISMLYLDENS'TVVLKNYQEMTVVGCGCR; or				

30

35

40

45

(g)

1	10	20	30	40
LYVDFQRDVGWDDWIVAPPGYQAFYCSGACQFPSAD				
35	50	60	70	
HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTELSA				
40	80	90	100	
ISMLYLDENEKVVLKNYQEMVVEGCGCR; or				

45

(h)

1	10	20	30	40
LYVDFS-DVGWDDWIVAPPGYQAFYCSGACQFPSAD				
50	50	60	70	
HFNSTN--H-AVVQTLVNNMNPBK-VPKPCCVPTELSA				
55	80	90	100	
ISMLYLDENEKVVLKKYQEMVVEGCGCR.				

12. The protein of claim 2 or polypeptide chain of claim 5 comprising a synthetic polypeptide chain of less than 200 amino acids.

5 13. The process of any one of claims 3, 4 and 8-11 wherein the protein comprises a synthetic polypeptide chain of less than 200 amino acids.

10 14. The process of any one of claims 3, 4 and 8-12 wherein the host cell is a prokaryotic or eukaryotic host cell, for example E. coli, bacillus, yeasts or animal cells (e.g. CHO or myeloma).

15 15. The protein of claim 2 or 12 or polypeptide chain of claim 5 or 12 wherein the protein or polypeptide is unglycosylated.

20 16. The process of any one of claims 3, 4 and 8-13 wherein the protein or polypeptide is unglycosylated.

17. An osteogenic device comprising a matrix in which is disposed the protein or polypeptide chain as defined in any one of the preceding claims.

25 18. An osteogenic device for implantation in a mammal, the device comprising: (a) a biocompatible, *in vivo* biodegradable matrix defining a scaffold of dimensions sufficient to permit the attachment, proliferation and differentiation of migratory progenitor cells from the body of said mammal; and (b) the protein or polypeptide as defined in any one of the preceding claims disposed in said matrix and accessible to said cells.

19. The device of claim 17 or 18 wherein said matrix comprises close-packed particulate matter having a particle size within the range of 70 to 850 μm (e.g. 70 to 420 μm).

25 20. The device of any one of claims 17-19 wherein said matrix comprises: (a) allogenic bone, e.g. demineralized, protein extracted, particulate, allogenic bone, (b) demineralized, protein extracted, particulate, deglycosylated xenogenic bone, (c) demineralized, protein extracted, particulate, xenogenic bone treated with HF or a protease; (d) materials selected from collagen, hydroxyapatite, calcium phosphates (e.g. tricalcium phosphate) and polymers comprising glycolic acid and/or lactic acid monomers, (e) a shape-retaining solid of loosely adhered particulate material e.g. collagen, (f) a porous solid or (g) masticated tissue, e.g. muscle.

21. The device of any one of claims 17-20 disposed within the marrow cavity of allogenic bone.

35 22. The device of any one of claims 17-21 for use in therapy, e.g. for inducing local cartilage and/or endochondral bone formation in a mammal by implanting the device in a mammal at a locus accessible to migratory progenitor cells.

40 23. Use of the protein or polypeptide as defined in any one of the preceding claims for the manufacture of: (a) a device according to any one of claims 17-22 or (b) a medicament, the medicament or device being for inducing local cartilage and/or endochondral bone formation in a mammal by implanting the device in a mammal at a locus accessible to migratory progenitor cells for periodontal treatment, cartilage repair and for the treatment of osteoarthritis or to correct non-union fractures, acquired or congenital craniofacial and other skeletal or dental anomalies.

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50

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FIG. 1-1

FIG. 1-2

FIG. 1-3

FIG. 1-4

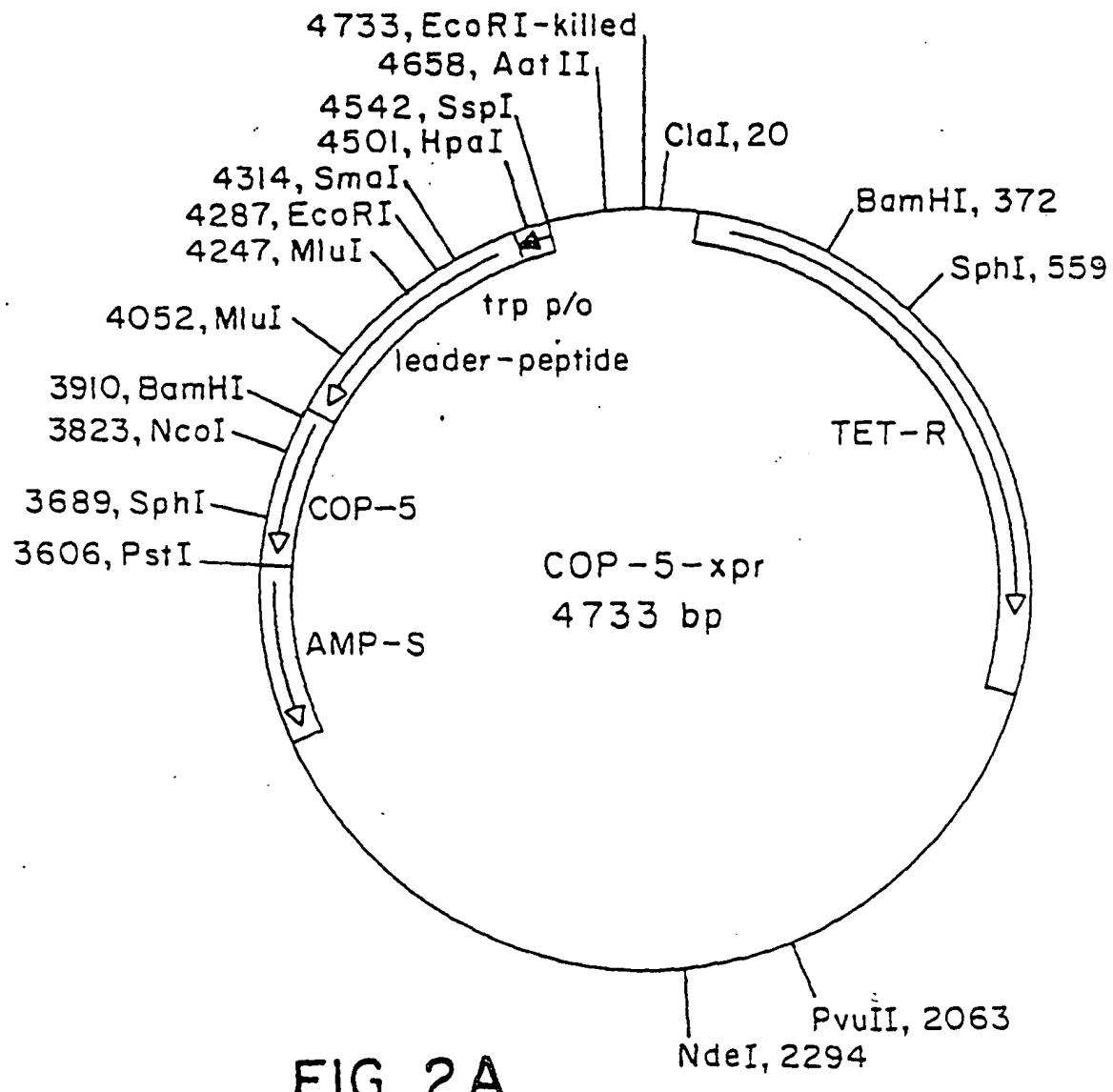


FIG. 2A

FIG. 2 B-1

COP-5 fusion protein

10 20 30 40 50
 ATGAAAGCAATTTCGACTGAAAGGTTCACTGGACAGAGATCTGGACTC
 M K A I F V L K G S L D R D L D S
 BglIII .

 60 70 80 90 100
 TCGTCTGGATCTGGACGGTACCGACCACAAAGACCTGCTGATCACC
 R L D L D V R T D H K D L S D H

 110 120 130 140 150
 TGGTTCTGGTCGACCTGGCTCGTAACGACCTGGCTCGTATCGTTACTCCC
 L V L V D L A R N D L A R I V T P
 Sall Sma

 160 170 180 190 200
 GGGTCTCGTTACGTTGGATCTGGAATTATGGCTGACAACAAATTCAA
 G S R Y V A D L E F M A D N K F N
 I EcoRI

 210 220 230 240 250
 CAAGGAACAGCAGAACGGCGTTACGAGATCTTGCACCTGCCAACCTGA
 K E Q Q N A F Y E I L H L P N L
 MluI BglIII BspMI+

 260 270 280 290 300
 ACGAAGAGCAGCGTAACGGCTTCATCAAAGCTTGAAGGATGAGCCCTCT
 N E E Q R N G F I Q S L K D E P S
 HindIII

 310 320 330 340 350
 CAGTCTGCGAATCTGCTAGCGGATGCCAAGAAACTGAACGATGCCAGGC
 Q S A N L L A D A K K L N D A Q A
 NheI FspI

 360 370 380 390 400
 ACCGAAATCGGATCAGGGCAATTATGGCTGACAACAAATTCAACAAAGG
 P K S D Q G Q F M A D N K F N K

 410 420 430 440 450
 AACAGCAGAACGGCGTTACGAGATCTTGCACCTGCCAACCTGAACGAA
 E Q Q N A F Y E I L H L P N L N E
 MluI BglIII BspMI+

 460 470 480 490 500
 GAGCAGCGTAACGGCTTCATCAAAGCTTGAAGGATGAGCCCTCTCAGTC
 E Q R N G F I Q S L K D E P S Q S
 HindIII

FIG. 2B-2

510	520	530	540	550
TGCAGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGCACCGA				
A	N	L	L	A
D A K K L N D A Q A P				
NheI				
560	570	580	590	600
AGGATCCTAATGGGCTGTACGTCGACTTCAGCGACGTGGCTGGGACGAC				
K	D	P	N	G
L Y V D F S D V G W D D				
BamHI				
610	620	630	640	650
TGGATTGTGGCCCCACCAGGCTACCAGGCCTTCTACTGCCATGGCGAATG				
W	I	V	A	P
P P G Y Q A F Y C H G E C				
StuI				
660	670	680	690	700
CCCTTCCCGCTAGCGGATCACTCAACAGCACCAACCACGCCGTGGTGC				
P	F	P	L	A
D H F N S T N H A V V				
NheI				
710	720	730	740	750
AGACCCTGGTGAACTCTGTCAACTCCAAGATCCCTAAGGCTTGCTGCGTG				
Q	T	L	V	N
S V N S K I P K A C C V				
MstII				
760	770	780	790	800
CCCAACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATGAGAA				
P	T	E	L	S
A I S M L Y L D E N E K				
SphI				
810	820	830	840	850
GGTGGTGCTGAAGAACTACCAGGAGATGGTAGTAGAGGGCTCCGGCTGCC				
V	V	L	K	N
Y Q E M V V E G C G C				
PfIMI				
860				
GCTAACTGCAG				
R	*			
PstI				

FIG. 3A

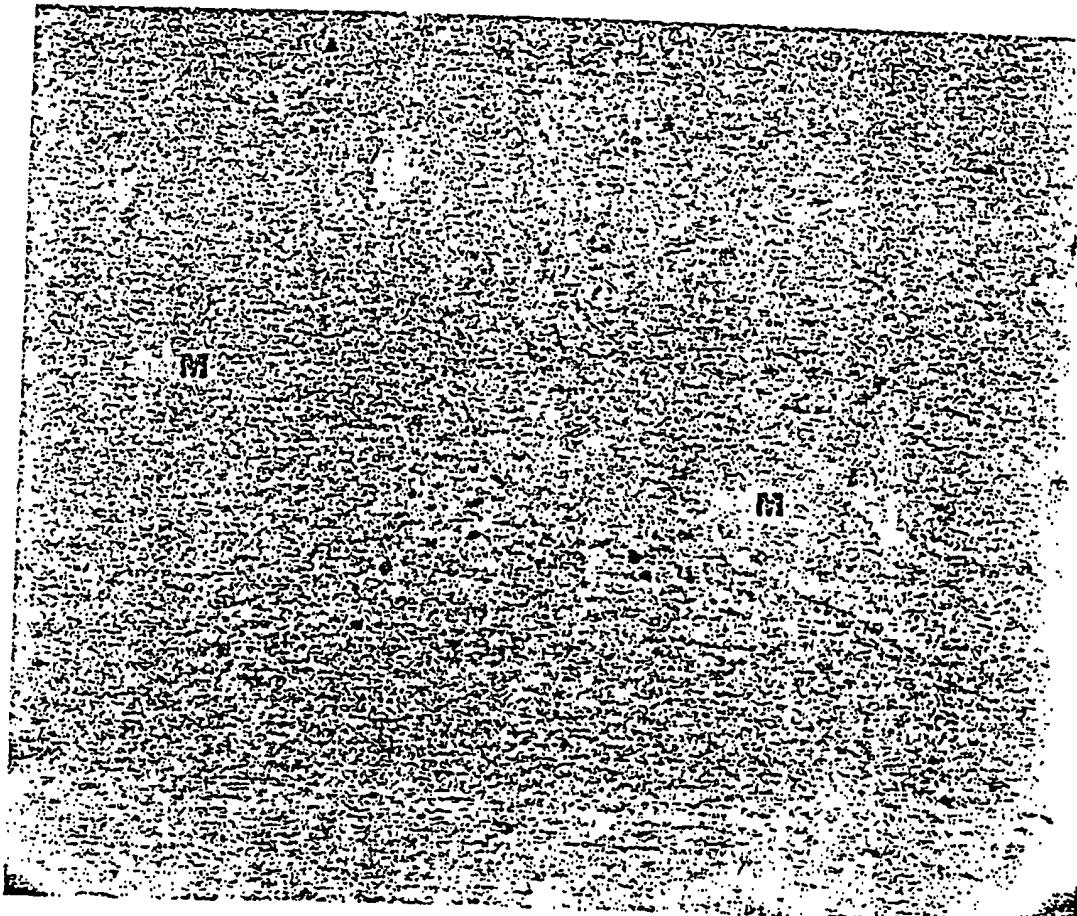


FIG. 3B

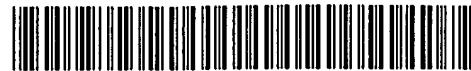


FIG. 4

10	20	30	40	50
GATCCTAATGGGCTGTACGTGGACTTCCAGCGCGACGTGGGCTGGGACGA				
D P N G L Y V D F Q R D V G W D D				
60	70	80	90	100
CTGGATCATGGCCCCCGTCGACTTCGACGCCACTACTGCTCCGGAGCCT				
W I I A P V D F D A Y Y C S G A				
110	120	130	140	150
GCCAGTTCCCTCTGCGGATCACTAACAGCACCAACCACGCCGTGGTG				
C Q F P S A D H F N S T N H A V V				
160	170	180	190	200
CAGACCCTGGTGAACAAACATGAACCCCGGCAAGGTACCCAAGCCCTGCTG				
Q T L V N N M N P G K V P K P C C				
210	220	230	240	250
CGTCCCCACCGAGCTGTCCGCCATCAGCATGCTGTACCTGGACGAGAATT				
V P T E L S A I S M L Y L D E N				
260	270	280	290	300
CCACCGTGGTGGTGAAGAACTACCAAGGAGATGACCGTGGTGGCTGCAGG				
S T V V L K N Y Q E M T V V G C G				
310				
TGCCGCTAACTGCAG				
C R *				



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(11) EP 1 221 484 A3

(19)

EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
18.09.2002 Bulletin 2002/38

(51) Int Cl.⁷: **C12N 15/12, C07K 14/51,**
A61K 38/18, A61L 27/22,
A61L 27/54

(43) Date of publication A2:
10.07.2002 Bulletin 2002/28

(21) Application number: 01201555.8

(22) Date of filing: 07.04.1989

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(30) Priority: 08.04.1988 US 179406
15.08.1988 US 232630
23.02.1989 US 315342

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
96200044.4 / 0 723 013
89904959.7 / 0 372 031

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(54) Biosynthetic osteogenic proteins and osteogenic devices containing them

(57) Disclosed are 1) osteogenic devices comprising a matrix containing osteogenic protein and methods of inducing endochondral bone growth and cartilage in mammals using the devices; 2) amino acid sequence data, amino acid composition, solubility properties,

structural features, homologies and various other data characterizing osteogenic proteins, 3) methods of producing osteogenic proteins using recombinant DNA technology, and 4) osteogenically and chondrogenically active synthetic protein constructs.

FIG. 1-1		:VGL DPP OPI CBMP2		:CBMP3		beta-		TGF-		:MIS		alpha::		consensus	
COP1	COP5	a)	b)	(bov)Inhibin		a)	b)	1	2	3	beta	Inhib	alpha	choices	
COP3	COP7														
COP4		C	C	C	C	C	C	C	C	C	C	C	C	C	C
<P	P	P	P	K	R	K	R	R	A	C	A	H			
<N	N	N	N	R	H	H	H	R	K	Q	R	R			
<G	G	G	G	H	S	E	P	S	Y	Q	P	V	E	A	
L	L	L	L	L	L	L	P	L	F	F	L	L	E	L	
Y	Y	Y	Y	Y	Y	Y	Y	K	F	F	Y	S	N		y,f
V	V	V	V	V	V	V	V	V	V	V	I	V	V		v,i
D	D	D	D	E	D	S	D	D	S	D	D	D	S	S	d,e,s
F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	f
Q	Q	S	S	S	K	S	R	S	A	K	R	R	Q	Q	k,r,s
R	R														
D	D	D	D	D	D	D	D	D	D	D	D	E	E	E	d
V	V	V	V	V	V	V	G	G	I	I	L	L	L	L	v,1,i
G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	g
W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	w
D	D	D	D	D	D	D	D	D	D	D	D	E	E	E	q,n,d,e,s
D	D	D	D	D	D	D	D	D	D	D	D	R	R	R	d,e,n
W	W	W	W	W	W	W	W	W	W	W	W	R	R	R	w
I	I	I	I	I	I	V	I	I	I	I	I	V	V	V	i,v
I	V	V	V	V	V	I	V	I	V	I	V	V	V	V	i,v
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	a,s
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	p
V	P	P	P	P	P	Q	L	G	P	S	T	E	E	E	g,q,p*
D	G	G	G	G	G	G	G	G	G	G	K	F	S	S	g,s
F	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	y,f
D	Q	Q	Q	Q	Q	H	H	H	H	H	H	H	H	H	h,d,n,q,y*
A	A	A	A	A	A	M	D	V	A	A	A	A	A	A	a

FIG. 1-2

FIG. 1-3

N	N	N	S	:	S	N	F	S	:	A	R	Q	T	T	Q	S	:	n,s*
M	M	M	V	V	:	I	N	V	N	G	G	H	I	I	R	L	:	i,v,l,
N	N	N	N	N	:	E	N	N	H	S	N	N	N	N	G	L	:	n
P	P	P	S	S	:	P	P	S	P	P	G	E	E	A	P	G	:	p,s,a
G	G	G	S	S	:	E	E	S	F	F	A	S	S	P	C	C	:	g,e
K	K	K	K	K	:	Q	K	T	K	A	S	A	S	C	C	C	:	k,q,t,s
V	V	V	I	I	:	I	V	P	A	C	S	S	S	P	V	P	:	i,v,l
P	P	P	P	P	:	P	P	P	A	C	C	C	C	C	V	P	:	p,a,s
P	K	K	K	K	:	L	K	P	K	A	C	C	C	C	V	P	:	a,p,s
C	C	C	A	A	:	P	A	C	K	A	C	C	C	C	V	P	:	c,c
C	V	V	C	C	:	C	C	C	C	C	C	C	C	C	V	P	:	v,a,1
V	P	P	V	P	:	V	V	P	P	P	P	P	P	P	V	P	:	p,s,a
P	T	T	E	E	:	P	T	T	T	T	T	T	T	T	T	T	:	t,e,g
E	L	L	E	E	:	K	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	:	g,e,d,k*
S	S	S	S	S	:	M	M	M	M	M	M	M	M	M	M	M	:	l,m
A	A	S	A	S	:	S	S	S	S	S	S	S	S	S	S	S	:	s,e,d
I	I	I	I	I	:	I	I	I	I	I	I	I	I	I	I	I	:	a,s,p,t
S	S	I	S	I	:	S	S	S	S	S	S	S	S	S	S	S	:	i,l,m,v
M	M	I	M	I	:	M	M	M	M	M	M	M	M	M	M	M	:	s,a,p,t
L	Y	M	L	M	:	L	L	L	L	L	L	L	L	L	L	L	:	v,m,i,l
D	D	L	D	L	:	F	F	F	F	F	F	F	F	F	F	F	:	l,v,i
E	E	D	E	D	:	D	D	D	D	D	D	D	D	D	D	D	:	y,f
E	E	E	E	E	:	N	N	N	N	N	N	N	N	N	N	N	:	E,l,y
N	N	N	N	N	:	N	N	N	N	N	N	N	N	N	N	N	:	d,e,
N	N	N	N	N	:	N	N	N	N	N	N	N	N	N	N	N	:	n,q,y*
E	E	E	E	E	:	N	N	N	N	N	N	N	N	N	N	N	:	e,d,s,t

FIG. 1-4

K	K	K	K	;	N	T	N	K	:	N	N	N	P	:	I	G	;	n,k
V	V	V	V	;	V	V	V	V	:	V	I	I	V	I	S	G	;	v,i
V	V	V	V	;	V	V	I	V	:	V	I	V	K	V	G	Y	;	v,i
L	L	L	L	;	L	L	L	K	:	L	K	K	E	Q	S	F	;	l,
K	K	K	K	;	K	K	K	K	:	K	K	R	D	V	K	S	;	k,r
N	N	N	N	;	H	N	Y	Y	:	V	Y	I	Q	P	Y	F	;	n,d,k*
Y	Y	Y	Y	;	Y	Y	Y	Y	:	P	Q	P	S	P	E	K	;	y
Q	Q	Q	Q	;	E	Q	R	Q	:	TVP	;	;	;	;	;	;	;	q,e,p,r
E	E	E	E	;	N	E	N	D	E	N	N	N	N	N	N	N	;	n,d,e
M	M	M	M	;	M	M	M	M	;	M	M	M	M	I	M	L	;	m
V	V	V	V	;	V	V	V	V	;	T	I	I	V	V	V	L	;	v,i,t,a
V	V	V	V	;	V	V	V	V	;	V	V	V	V	V	V	L	;	v
E	E	E	E	;	D	V	R	E	;	E	E	E	R	K	T	Q	;	e,d,r,k,
G	G	G	G	;	E	G	A	G	;	S	E	E	S	S	E	H	;	g,a,s,e
C	C	C	C	;	C	C	C	G	;	C	C	C	C	C	C	C	;	c
G	G	G	G	;	G	G	G	G	;	H	G	G	K	K	G	A	;	g,h
C	C	C	C	;	C	C	C	C	;	C	C	C	S	S	C	A	;	c
R	R	R	R	;	R	R	R	H	;	R	S	S	A	S	R	I	;	r,h,s,a,



EUROPEAN SEARCH REPORT

Application Number
EP 01 20 1555

DOCUMENTS CONSIDERED TO BE RELEVANT			
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		-/-	
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	14 May 2002	van de Kamp, M	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			



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The present search report has been drawn up for all claims			
Place of search THE HAGUE	Date of completion of the search 14 May 2002	Examiner van de Kamp, M	
CATEGORY OF CITED DOCUMENTS		I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
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EUROPEAN SEARCH REPORT

Application Number
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			TECHNICAL FIELDS SEARCHED (Int.Cl.7)												
<p>The present search report has been drawn up for all claims</p> <table border="1"> <tr> <td>Place of search THE HAGUE</td> <td>Date of completion of the search 14 May 2002</td> <td>Examiner van de Kamp, M</td> </tr> <tr> <td colspan="3">CATEGORY OF CITED DOCUMENTS</td> </tr> <tr> <td colspan="3"> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document </td> </tr> <tr> <td colspan="3"> T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document </td> </tr> </table>				Place of search THE HAGUE	Date of completion of the search 14 May 2002	Examiner van de Kamp, M	CATEGORY OF CITED DOCUMENTS			X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document		
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CATEGORY OF CITED DOCUMENTS															
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T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document															

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14-05-2002

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